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High Mobility Group Protein 1 (HMGB1) And Its Role As A Global Transcription Regulator In Response To Temperature Fluctuations In The Annual Killifish *Austrofundulus limnaeus*

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High Mobility Group Protein 1 (HMGB1) And Its Role As A Global
Transcription Regulator In Response To Temperature Fluctuations In
The Annual Killifish *Austrofundulus limnaeus*

by

Victoria Martin Alla

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
In
Biology

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Abstract

As a study organism, annual killifish (*Austrofundulus limnaeus*) provide a well suited study system for examining the effects of environmental temperature fluctuations at the cellular level. *A. limnaeus* persist in the harsh high desert climate of the Maracaibo Basin, Venezuela where they live in small, ephemeral freshwater pools. Temperatures in these waters can vary as much as 20°C daily and reach maximums of over 40°C due to the semi-arid climate. Previous cDNA microarray studies on killifish revealed the mRNA pattern for High Mobility Group Protein 1 (HMGB1) to be strongly affected by temperature perturbations. Specifically, peaks in *hmgb1* transcript abundance were negatively correlated with temperature during temperature cycling, and experienced over a 10 fold difference in expression in response to the temperature cycle. Using the same temperature cycling experimental setup, this study's aim was three-fold: (1) to characterize the total amount of HMGB1 protein in adult male killifish livers, (2) to describe the subcellular localization of the HMGB1 protein in adult male killifish livers and (3) to sequence the 5' upstream region of the *hmgb1* gene to identify possible stress responsive elements. We detected no significant difference in total HMGB1 protein levels as a consequence of temperature cycling. The data for subcellular localization of HMGB1 protein do not support a strong change in subcellular localization of the protein in response to temperature cycling; most of the HMGB1 protein is found in the cytoplasmic compartment in liver tissue. Although overall patterns of subcellular localization did not change significantly, we found a

significant difference between nuclear HMGB1 protein levels in temperature cycled fish versus control (constant temperature) fish. This could suggest a muting of the natural translocation of HMGB1 into the nucleus observed in control fish at around 9:00 at night. Finally, the upstream region of the *hgmb1* gene does reveal a number of putative stress responsive transcription factor binding sites.

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Introduction

Temperature Sensing and Thermal Biology

Organisms continuously experience a wide array of environmental stimuli throughout their life, and temperature is arguably one of the most significant environmental parameters for any organism. For ectotherms and endotherms alike, the detection and control of temperature is crucial to continued persistence and survival. In the words of Hochachka and Somero (2002), “temperature affects every aspect of an organism’s physiology, from the basic structures of the macromolecules that are responsible for catalysis and information processing to the rates at which chemical reactions occur” (Biochemical Adaptation pp. 290). Consequently, organisms employ diverse adaptive strategies in order to ensure their continued existence in a thermally heterogeneous environment. When it comes to thermal tolerance, eurythermal organisms can endure wide ranges of temperatures as well as extremes in absolute temperature on both short (hourly, daily) and long (seasonal, annual) time scales (Hochachka and Somero 2002). The annual killifish, *Austrofundulus limnaeus*, is a eurythermal freshwater fish that provides an exceptionally well-suited system in which to study short time-scale temperature effects on organismal physiology. In this thesis I explore the role of the high mobility group protein 1 (HMGB1) in supporting the exceptional eurythermal nature of adult *A. limnaeus*.

Background and Thermal Biology of *Austrofundulus limnaeus*

Austrofundulus limnaeus (killifish) are part of the family Rivulidae (order Cyprinodontiformes) which is distributed from southern Mexico into central Argentina (Hrbek and Larson 1999). Within this range, Rivulid species can be found in a wide array of habitats from seasonally dry savannahs to tropical rainforests. The genus *Austrofundulus* is split into seven extant species based on mitochondrial DNA data; *A. limnaeus* is spatially restricted to the eastern side of Lake Maracaibo, Venezuela (Hrbek et al. 2005). *A. limnaeus* is also defined as an “annual” member of the family Rivulidae. “Annual” refers to the fact that *A. limnaeus* inhabit temporary bodies of water and must employ a strategy that allows them to persist even when their ponds dry up. They do this by laying eggs that are capable of entering different states of developmental arrest termed “diapause” (Wourms 1972c). These diapausing embryos can endure extremes in salinity, oxygen concentration, and temperature (Machado and Podrabsky 2007; Podrabsky et al. 2007).

In these ephemeral ponds, killifish often encounter considerable fluctuations in temperature on both a daily and seasonal scale. With little canopy cover to temper the intense tropical sun, water temperature in these ponds can vary significantly on an hour-by-hour basis. Temperatures can fluctuate as much as 20°C on a daily basis and reach maximums of over 40°C due to the semi-arid climate in the Maracaibo Basin (Podrabsky et al. 1998). Although these

temperatures are not as extreme as some organisms endure (such as the hydrothermal vent worm *Alvinella pompejana* at 48.6°C (Biochemical Adaptation pp. 360)) they do represent a significant physiological insult for vertebrates (Tomanek 2010). Killifish habitats are similar in thermal profile to those of rocky intertidal zones where residents can experience temperature changes of more than 20°C on a daily basis (Tomanek 2010). To cope with these thermal stresses, intertidal organisms active the heat shock response (HSR) as a biochemical coping strategy (Tomanek 2010). Additionally, these organisms are often found living very close to their maximum thermal thresholds (Tomanek 2010). As compared to *A. limnaeus*, other species of fish persist in much more thermally stable environments. For example, the critical thermal maximum (T_{crit} or the temperature at which an organism becomes incapacitated due to heat) for redband trout (*O. mykiss*) is 29.1°C and the T_{crit} for the atlantic salmon (*S. salar*) is 32.7°C (Strange 2010). When exposed to 30°C, seabream (*S. aurata*) experience 20% mortality within 10 days (Feidantsis et al. 2009). Therefore, due to the extreme environmental conditions which both the adult killifish and embryos experience, this species is an excellent study organism for investigating how vertebrate organisms cope with a highly thermally variable environment.

Molecular Biology of HMGB1:

HMGB1 was first discovered over 30 years ago and identified as a protein with a high electrophoretic mobility (Goodwin and Sanders 1973). The protein has

a weight of 25 kDa but migrates to a position of 30 kD on a SDS-PAGE gel, perhaps because of the high number of positively charged amino acids contained in the protein (Bianchi and Manfredi 2007). Although the exact mechanism is still unclear, HMGB1 is essential for vertebrate life; HMGB1 knockout mice (*hmgb1*^{-/-}) die soon after birth due to hypoglycemia (Calogero et al. 1999). However, cell lines that are deficient in HMGB1 (derived from *hmgb1*^{-/-} mice) are able to survive; suggesting that HMGB1 is not a structural component of chromatin but instead mediates accessibility to the nucleosomally packaged DNA (Calogero et al. 1999). Research indicates that the HMG box superfamily most likely evolved over 1,000 million years ago (Laudet et al. 1993). Among mammals there is 99% amino acid sequence identity, indicating that HMGB1 is highly conserved and that each residue is under strong selective pressure (Sessa and Bianchi 2007). Genes that are orthologous or paralogous to mammalian *hmgb1* are found in all other multicellular organisms. Indeed the earliest HMGB proteins exist in the simplest Metazoans; specifically sponges (Sessa and Bianchi 2007).

Function of HMGB1 protein

In mammals, the *hmgb1* gene codes for a 215 amino acid protein that contains three domains. Two are homologous, “L-shaped” domains (Hardman et al. 1995) which are responsible for DNA-binding and are referred to as the A box (76 amino acids) and the B box (74 amino acids) (Bianchi and Manfredi 2007, Weir et al. 1993). The third domain is an acidic tail containing 30 negatively

charged amino acids at the C terminal end (Wang et al. 2007). The HMGB1 protein does not recognize specific DNA sequences; instead it binds to specific DNA structures (such as four way junctions) as well as to the minor groove of DNA where it induces bends in the DNA (Boonyaratanakornkit et al. 1998). The HMGB1 molecule can increase its affinity for non-linear DNA's through regulation of its acidic tail. The acidic C-terminal tail folds back over the protein and interacts with the N-terminal region allowing only specific DNA structures the access needed to interact with the DNA binding domains (Wang et al. 2007). In this way it serves as an architectural factor and allows HMGB1 to physically interact with and modify DNA and facilitate many nuclear events such as transcription, replication, V(D)J recombination and DNA transposition (Bianchi and Manfredi 2004). Additionally, it allows other proteins to interact with the DNA such as p53, nuclear factor- κ B (NF- κ B), homeobox-containing proteins, recombination activating gene 1/2 (RAG1/2) protein, and steroid hormone receptors (Bianchi and Manfredi 2007). Through a series of photobleaching experiments, Bianchi and Manfredi (2004) illustrated that HMGB1 proteins "roam" the nucleus and do not show marked residential status. From these experiments they calculated that each nucleosome is visited on an average of every 2 seconds and the protein remains there for only for a few seconds (Bianchi and Manfredi 2004 and Phair et al. 2004). Thus, they concluded that HMGB1 acts as a "chromatin chaperone" propelled simply by Brownian motion (Bianchi and Manfredi 2004).

While there is a clear role for HMGB1 in nuclear function, the protein also operates in the cytoplasm and even in the extracellular space (Dumitriu et al. 2005). Additionally, HMGB1 can also be expressed on the plasma membrane of certain cells in a form known as amphoterin (Dumitriu et al. 2005). In this membrane-associated form, amphoterin is involved with neurite outgrowth, tumor cell metastasis and smooth muscle cell chemotaxis (Dumitriu et al. 2005). HMGB1 reaches the extracellular space via passive mechanisms in the case of necrotic cells; or via active secretion, in the case of certain immune cells (Dumitriu et al. 2005).

In dying or damaged cells, HMGB1 “leaks” only from those cells experiencing necrotic death, and not from cells dying via apoptosis. Apoptotic cells tightly bind HMGB1 to the chromatin in the nucleus (Scaffidi et al. 2002). HMGB1’s role in the necrotic scenario involves triggering the inflammatory response in answer to the trauma, poison, or infection that has damaged the cells (Scaffidi et al. 2002). Inflammation is a critical reaction to tissue injury in mammals and other vertebrates, and HMGB1 plays a critical role in communicating this response to neighboring cells (Scaffidi et al. 2002, Wang et al. 1999). HMGB1 plays a key role as an inflammatory cytokine that acts as a late mediator of sepsis. When stimulated with lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNF- α) for 18 hours, *in vitro* murine (mouse) macrophages produce HMGB1 in a time dependant manner. Release of HMGB1 starts 8 hrs post insult and peaks at 24-32 hrs post insult (Wang et al. 1999). Since HMGB1 is a “late” player in the inflammation cascade, it provides a tempting therapeutic target.

In fact, anti-HMGB1 antibodies can protect against lethality caused by sepsis (Yang et al. 2004). In this experiment, researchers perforated the large intestines of live, anesthetized mice which induced sepsis via an intra-abdominal infection. Anti-HMGB1 antibodies administered 24 hrs after onset of sepsis reversed the lethality of the infection (Yang et al. 2004). This treatment provides a relevant therapeutic option for clinical sepsis.

Subcellular Localization

The subcellular localization of HMGB1 appears to be regulated by a number of different factors, some of which are canonical, while others are unique. Nuclear localization is accomplished via two nuclear localization signals (NLS's) from amino acids 27-43 and 178-184 (Bonaldi et al. 2003). However, secretion of HMGB1 does not occur via the classical endoplasmic reticulum (ER)-Golgi signal peptide which typically results in cotranslation of the protein into the rough ER and eventually to secretion of the protein via exocytosis (Yang et al. 2010 and Gardella et al. 2002). In mammalian macrophages and monocytes, covalent modifications to HMGB1 cause translocations of the protein from the nucleus into the cytoplasm. HMGB1 can be modified in a number of ways including: acetylation, methylation, phosphorylation, poly (ADP)-ribosylation and oxidation (Bonaldi et al. 2003, Ito et al 2007, Youn and Shin 2006, Ditsworth et al. 2007, Hoppe et al. 2007). Due to HMGB1's relatively small size, it passively diffuses through nuclear pores and in rat and cow liver cells and there is more HGMB1 found in the cytoplasm than in

the nucleus (Mosevitsky et al. 1989). Conversely, in murine monocytes, the majority of the protein is located in the nucleus which suggests that nuclear import is much more effective than nuclear export in this species (Bonaldi et al. 2003). These cells can accumulate HMGB1 in the cytoplasm in two ways, by hyperacetylation of specific lysine residues that neutralize the positively charged NLS and thus block the protein's re-entry into the nucleus, and by interaction of two nuclear export signals with the chromosomal region maintenance 1 (CRM1) exportin (Bonaldi et al. 2003). In human neutrophil cells, HMGB1 can be monomethylated at lysine 42 which induces a conformational change in the protein that weakens its DNA binding ability. This results in a passive accumulation of the protein in the cytoplasm (Ito et al. 2007). Phosphorylation of HMGB1 in murine macrophages is performed by the classical protein kinase C (cPKC) and occurs mainly on six serine residues that are located near the NLSs (Oh et al. 2009, Youn and Shin 2006). This phosphorylation blocks the protein's movement back into the nucleus and results in increased cytoplasmic residency (Youn and Shin 2006).

Subcellular localization of HMGB1 can also be affected by cellular stress. When monocytes experience DNA alkylating damage, the poly (ADP)-ribose polymerase (PARP) activates and helps translocate HMGB1 from the nucleus to the cytoplasm (Ditsworth et al. 2007). Although the exact mechanism is still unclear, PARP seems to act on the acidic tail of HMGB1 to cause its translocation (Ditsworth et al. 2007). It is possible that this translocation is used to prepare the cell to release HMGB1 in case of necrotic death, and thus propagate the

inflammatory cascade (Ditsworth et al. 2007). In cases of oxidative stress, three cysteines (23, 45 and 106) appear to play a role in conformational changes as well as localization of HMGB1 (Hoppe et al. 2006). Mutations in cysteine 106 cause a build up of HMGB1 in the cytoplasm; perhaps due to a direct interaction of cysteine 106 with the NLS. Although changes to cysteines 23 and 45 do not seem to mediate subcellular localization of HMGB1, they do experience conformational changes due to oxidation which could lead to regulation of HMGB1 (Hoppe et al. 2006).

Secretion of HMGB1

Active secretion of HMGB1 has only been identified thus far in human and mouse cells of the innate immune system (Wang et al. 1999). In human monocytes, when HMGB1 has achieved entrance and residency in the cytoplasm, it is then translocated into cytoplasmic organelles. These organelles seem to belong to a special subset of secretory lysosomes found within monocytic cells (Gardella et al. 2002). Additionally, HMGB1 may localize further to specialized subcompartments of these lysosomes. This hierarchical storage of HMGB1 could allow for tightly regulated exocytosis of the protein and thus confer strict control of the inflammatory response (Gardella et al. 2002). In the specific case of phosphorylation, secretion of HMGB1 is accomplished by a calcium dependant mechanism that has yet to be identified (Oh et al. 2009).

HMGB1 and Thermal Acclimation in *A. limnaeus*

Because of their unique life history, killifish provide significant insights into how organisms cope with temperature change at the gene expression level. Microarray gene expression profiling in adult liver tissue of *A. limnaeus* exposed to a variety of thermal acclimation regimens led to the current hypothesis “that constant and fluctuating environments elicit different transcriptional and likely physiological responses” (Podrabsky and Somero 2004). In this study, the fish were exposed to four temperature acclimation schemes: constant temperatures of 20, 26 and 37°C, and daily temperature cycling from 20-37°C designed to imitate the natural temperature changes experienced by *A. limnaeus* every day (Podrabsky et al 1998). Transcriptional responses to temperature were identified in less than 10% of the nearly 5000 cDNA elements printed on the array when a 2-fold change in gene expression was used as an arbitrary threshold for differential expression. One main finding of the study was that distinctive transcriptional responses are associated with constant versus fluctuating temperature regimes. For example, small heat shock proteins (Hsp22 and Hsp27) seem to respond to fluctuating temperatures while large Hsps (Hsp70 and Hsp90) react to constant high temperatures. This trend is mirrored in a variety of genes including ones involved in the maintenance of membrane integrity as well as ones implicated in modification of fatty acid saturation and biosynthesis. However, one of the most interesting results was the transcriptional expression profile of the *hmgb1* gene. Specifically, peaks in *hmgb1* transcript abundance were negatively correlated with temperature during

temperature cycling, and experienced over a 10 fold difference in expression in response to the temperature cycle. Additionally, this response is not affected by any endogenous circadian patterns. In Figure 1, Panel A represents the transcriptional response of fish that were held at a constant temperature of 26°C for two weeks; these samples display no circadian relationship. The data in Panel B are the raw transcript data for the fish experiencing daily temperature cycling for two weeks. Finally, in Panel C, the control data (Panel A) has been subtracted from the temperature cycling data (Panel B) in order to correct for any autogenous patterns from the control samples. Panel C shows a very strong relationship between temperature cycling and *hmgb1* transcript levels even when corrected for any circadian rhythms. Consistent with the temperature cycling data, transcript abundance increased and remained steady in response to constant cold (20°C) conditions and decreased and remained steady in response to constant warm conditions (37°C). Additionally, *hmgb1* was the only gene with a transcriptional response that was highly correlated with the temperature cycle over the entire 2-week acclimation period.

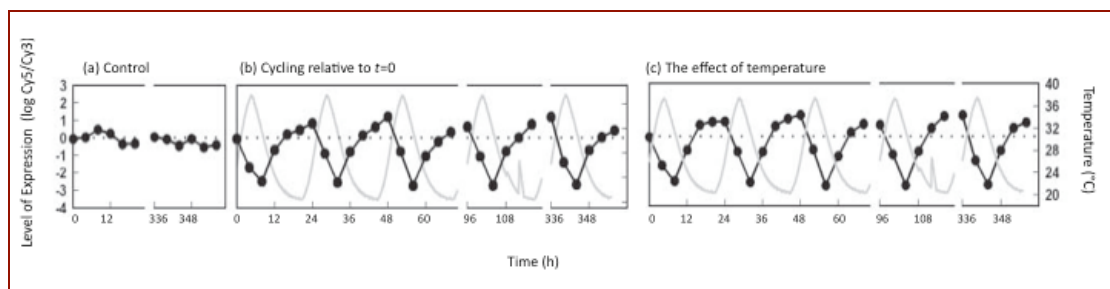


Figure 1. mRNA transcript profile for *hmgb1* gene in *A. limnaeus* in response to constant (a) and cycling temperatures (b,c). The dotted line in each graph represents a 1:1 ratio relative to the appropriate control. The temperature cycle is represented by the light grey line. The solid black line represents the mRNA transcript level.

Podrabsky and Somero (2004) developed a hypothesis that related the reported thermal stability of the mammalian HMGB1 protein with the transcriptional response observed in *A. limnaeus*. Circular dichroism (CD) studies show that HMGB1 protein begins to melt at 44.2°C, suggesting that it may be extremely sensitive to thermal denaturation at physiologically relevant temperatures (Ugrinova et al. 2009). A melting temperature of 44.2°C is low when compared with many other eukaryotic proteins such as heat shock proteins (Hsp's). CD studies of human heat shock proteins show that Hsp22 melts at ~58°C, Hsp25 melts at ~60°C and Hsp70 melts at ~60°C while common cellular proteins such as actin melts at ~57°C and lactate dehydrogenase melts at ~ 58° C (Kazakov et al. 2009, Morris et al. 2008, Carrigan et al. 2005, Bertazzon et al. 1990, Fields et al. 2002). Podrabsky and Somero (2004) hypothesized that when HMGB1 begins to melt, it disrupts the ability of the protein to “maintain the nucleoprotein complexes associated with transcription initiation and causes a global change in the rate of transcription.” However, this working hypothesis is based on changes in transcription of the gene, and has not been formally tested. Currently, the importance of the unique transcriptional response of the *hmgb1* gene in response to temperature fluctuations is unknown.

To date, there has been little work done on how the HMGB1 protein responds to environmental perturbations such as temperature fluctuations. The thermal tolerance of *A. limnaeus* and the large amount of existing transcriptional data

provides an ideal system in which to study the mechanics of this protein. In this thesis I explored the abundance and subcellular localization of the HMGB1 protein in response to temperature fluctuations. In addition, I have sequenced the promoter region of this gene in *A. limnaeus* in order to investigate possible regulatory control over the transcription of this gene. To this end I have formed four hypotheses that will be tested in this thesis:

1. The relative amount of HMGB1 protein will match the relative amount of *hmgb1* transcript in liver tissues
2. The relationship between *hmgb1* transcript and protein levels will remain the same for fish acclimated to a constant temperature as well as fish exposed to temperature fluctuations.
3. HMGB1 protein will be mainly localized in the nucleus under both control and experimental temperature treatments.
4. The underlying genetic structure of the *hmgb1* gene in *A. limnaeus* will be similar to other vertebrates and will contain stress responsive elements that may help explain the gene's transcriptional response to temperature.

Materials and Methods

Temperature Acclimation Protocol

To explore the effect of temperature on protein expression in *A. limnaeus*, we exposed adult male fish (~4 months of age) to two laboratory temperature regimes: a constant temperature of 26°C, or a daily cycling temperature regime from 20-37°C as described in Podrabsky and Somero (2004). Fish used in this experiment were raised from a laboratory stock that is the same as the one used for the 2004 experiments by Somero and Podrabsky. This stock has been maintained for many generations at 26-28°C under established conditions (Podrabsky 1999). Fish were fasted for 16 hr prior to the start of the experiment. Temperature cycling (t=0) was started at 9:00 AM and a temperature of 26°C. Fish (n=3-4) were sacrificed at the appropriate time point (see below for details). All procedures used in these experiments were reviewed and approved by the PSU IACUC in accordance with the ethical treatment of research animals.

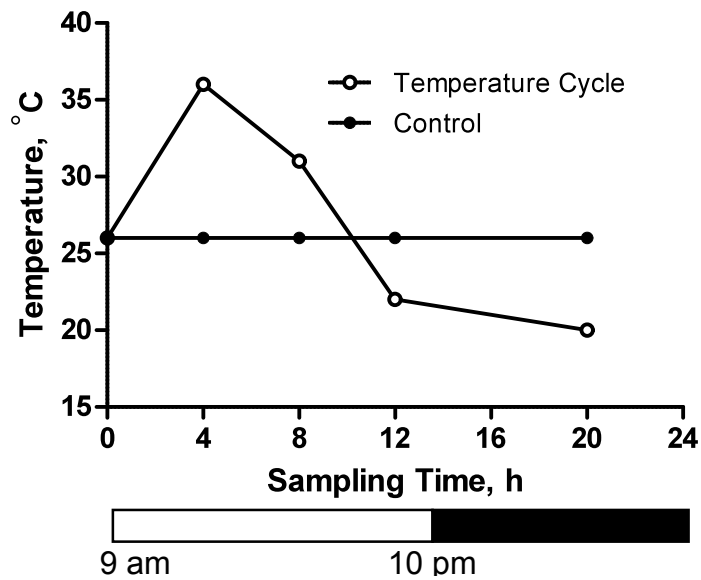


Figure 2. Graph represents sampling timepoints, temperature and light/dark cycle. Closed circles represent sampling times and temperatures for control fish (26°C). Open circles represent sampling times and temperatures for experimental “temperature cycle” fish (20-37°C). Bar at bottom represents 14 hour light/dark cycle for fish (lights come on at 8am).

Western Blot Processing

For western blot analysis of total liver HMGB1 protein levels, four fish were removed from the control and temperature cycling acclimation tanks at 0, 4, 8, 12, and 20 hr after the commencement of temperature cycling and immediately flash-frozen in liquid nitrogen (Figure 2). Whole fish were stored at -80°C for 2 weeks prior to tissue extraction and homogenization. Liver samples were homogenized with a ground glass tissue grinder in 3 volumes by weight of RIPA cell lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% Nonident-P40, 0.25% deoxycholate) to which a protease inhibitor cocktail was added (MiniComplete; Roche Diagnostics). Homogenates were sonicated (Sonics VCX 130, amplitude =

50) and then subjected to centrifugation for 10 min at 14,000 x g at 4°C with a fixed angle rotor, after which the supernatant was retained and the pellet discarded.

For analysis of sub-cellular fractions of liver HMGB1 protein levels, three fish were removed from each acclimation tank at 0, 4, 12, and 20 hr after the start of temperature cycling and placed directly into an ice/water bath. This treatment quickly anaesthetizes the fish without the use of drugs. The fish were then immediately sacrificed by cervical translocation and subcellular fractionation was carried out according to Rendell et al. (2006). Liver tissue was harvested and immediately placed into 9 volumes (by weight) of ice-cold TEGD buffer (50 mM Tris, 1.5 mM EDTA, 1 mM DTT, 30% glycerol, 2 mM protease inhibitor; pH 7.9) and homogenized on ice with 10 passes in a Teflon glass homogenizer. The homogenate was centrifuged at 1000 x g for 10 min at 4°C with a fixed angle rotor to produce a crude nuclear pellet and a crude cytoplasmic supernatant. The nuclear pellet was then washed twice in TMDS buffer (50 mM Tris, 5 mM MgCl₂, 250 mM sucrose, 1 mM DTT; pH 7.4) and subjected to centrifugation at 750 g for 10 min at 4°C after each wash. The pellet was resuspended in 500 µl of TEGD + KCl (TEGD buffer with 0.6 M KCl; pH 7.4) via intermittent vortexing over a period of 60 min. During this resuspension the pellet was incubated on ice. The lysed nuclear fractions were then subjected to centrifugation at 12,000 x g for 20 min at 4°C with a fixed angle rotor, and the supernatant retained. All samples were stored at -80°C until use for western blot analysis.

The total protein content of all homogenates (total lysate, cytoplasmic lysate and nuclear lysate) was determined with the Micro-BCA Protein Assay Kit (Pierce Scientific). 30 µg of total protein from whole liver lysate samples and 20 µg of total protein from subcellular samples were subjected to SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels. Separated proteins were electroblotted onto nitrocellulose membranes (Whatman) at 30V overnight at 4°C. Membranes were blocked in a solution of phosphate-buffered saline (PBS; 8g NaCl, 0.2g KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄; pH 7.4) and 5% (w/v) of non-fat dry milk (Nestle Carnation). Blots were then washed three times for 5-min each in PBS and 0.1% (v/v) Tween-20 (CalBiochem) prior to incubation in primary antibody (GenWay Biotech Cat. #10-663-46237; 1:1000 dilution in PBS containing 2.5% bovine serum albumin (BSA)) overnight at 4°C with constant shaking. After primary antibody incubation blots were washed three times for 5-min each in PBS and 0.1% Tween-20. Blots were then incubated in horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology; diluted to 1:5000 in PBS containing 2.5% BSA) for 1 hr at room temperature. Blots were then washed five times for 5-min each in PBS containing 0.1% Tween-20. All blots were exposed to Immobilon Western Chemiluminescent HRP Substrate (Millipore) for 5 min. Images of blots were captured by exposing the membrane to a charged-coupled device camera and processed with AlphaEase FC software (Cell Biosciences, Santa Clara, CA). All samples were standardized to a single sample that was present on each blot.

Immunohistochemistry

For determining sub-cellular localization of HMGB1 protein using immunohistochemical analysis, fish were sampled at 0, 4, 8, 12, and 20 hr after commencement of temperature cycling, and were placed directly into an ice/water bath. The fish were then immediately sacrificed by cervical translocation and liver tissue was harvested. The tissue was placed into cassette chambers and submerged in a solution of 10% zinc formalin (Protocol) for 72 h. The fixed tissue was embedded in paraffin using a Tissue Tek VIP Automatic Tissue Processor in the Oregon Health Sciences University's West Campus Morphology Core Lab. Liver tissues were sectioned into 5-8 μm sections using a microtome. Sections were mounted on Superfrost Plus (Fisher) glass slides treated electrostatically to facilitate adherence of the sectioned tissue. Tissue sections were deparaffinized with xylene for 5 min and transferred to 100% ethanol. Sections were then rehydrated and transferred into PBS by incubation in a series of solutions of decreasing ethanol concentrations (100%, 95%, 70% ethanol, PBS; each for 5 min). Heat-induced antigen retrieval was performed in 10 mM citrate buffer by boiling in a pressure cooker for 5 min followed by a 20 min "cool down" period. Endogenous peroxidases were blocked by immersing slides in a 0.3% hydrogen peroxide solution for 30 min. Slides were then washed in PBS (three 5 min washes). Blocking and antibody incubation were carried out using the Vector Labs Impress Kit (Vector Labs) according to the manufacturer's instructions at room temperature

unless otherwise noted. In brief, the slides were blocked with 2.5% normal horse serum for 30 min. Slides were incubated overnight at 4°C in HMGB1 primary antibody (Genway Biotech, Cat # 10-663-46237, 1:200) diluted in 2.5% normal horse serum. The slides were then washed in PBS (three 5 min washes) and incubated in Impress reagent for 30 min, followed by another three 5 min washes in PBS. The slides were then stained with 3,3'-Diaminobenzidine (DAB) solution for 2 min using the Vector DAB Kit (Vector Labs) according to manufacturer's instructions. The DAB reaction was stopped by rinsing the slides in reagent grade water (Nanopure, Millipore) for 5 min. Slides were counterstained with hematoxylin (Fisherbrand Harris Modified Hematoxylin, Fisher Scientific) for 30 s, dehydrated by immersion in 70% and then 100% ethanol followed by xylene and finally mounted onto glass slides using Permount Mounting Medium (Fisher Scientific).

5' Upstream Sequencing

The DNA sequence of the putative promoter region of the *hmgb1* gene in *A. limnaeus* (the region upstream of the transcription start site) was identified using the Universal GenomeWalker Kit (Clontech) according to the manufacturer's instructions. Genomic DNA was isolated from white muscle tissue using a Qiagen DNEasy kit according to the manufacturer's instructions. The isolated DNA was re-purified by extraction with phenol and chloroform, and precipitated by the addition of 2.5 volumes ethanol and 0.1 volumes of 3 M sodium acetate, prior to

resuspension in 20 µl of TE buffer (10mM Tris, 0.1 mM EDTA; pH 7.5). The isolation of high molecular weight DNA was confirmed via gel electrophoresis on a 1% agarose gel stained with ethidium bromide. Four restriction enzymes (*PvuII*, *EcoRV*, *ScaI* and *DraI*) were used to generate blunt-end fragments of various lengths, thereby creating four distinct restriction libraries. Adaptor primers (universal walking adaptors) were then ligated onto each of these “libraries” at both ends. Each library was used as a template in two subsequent polymerase chain reactions (PCR). The first PCR reaction (Table 1; primers AP1 and GSP1), employed a touchdown PCR protocol with the first 7 cycles consisting of 25 s at 94°C and 3 min at 72°C followed by 32 cycles of 25 s at 94°C and 3 min at 67°C and a final extension of 67°C for 7 min. Primers for this first reaction consisted of one adaptor primer that is complementary to the universal adaptor (Table 1; primer AP1) and one primer that was designed specifically to identify the 5’ end of the *A. limnaeus* cDNA sequence (Table 1; primer GSP1). This sequence is based on a previously reported mRNA transcript sequence for *hmgb1* in *A. limnaeus* (Podrabsky and Somero, 2004, GenBank accession number CK817293.1). The second (nested) PCR reaction was performed using 1 µl of the diluted (1:50) product of the first PCR reaction as a template. In this second PCR reaction, a set of nested adaptor (Table 1; primer AP2) and gene-specific (Table 1; primer GSP2) primers was used to generate PCR products specific to the *hmgb1* sequence. The nested PCR reactions utilized the same cycling protocol as reported above for the primary PCR reaction except that the first and second steps used 5 and 20 cycles,

respectively. PCR products were cloned into a pGEM T-Easy Vector (Promega) according to manufacturer's instructions. The clones were then transformed into DH10B competent cells and grown on blue/white screening LB plates (containing 50 µg/l carbenicillin, 100 mM IPTG and 20 mg/ml X-Gal) at 37°C for 48 hrs. White colonies were picked and grown in liquid LB broth containing 100 µg/l ampicillin overnight at 37°C. The plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. The plasmids were sequenced via Sanger sequencing using standard SP6 and T7 primers at the Oregon Health and Science University-Molecular Microbiology and Immunology (OHSU-MMI) Research Core Facility.

Name	Sequence 5' to 3'
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
GSP1	AGGTCCTCAAACCTTGCCCTTCTCCATT
GSP2	GCTCTGAGCACTTCTTGGAGAACTCG

Table 1. Sequences of the primers used in GenomeWalker protocol as outlined above.

Calculations and Statistics:

All western blot data are presented as “% of Time = 0.” To accomplish this, I established an average for the fish sampled at time = 0. I then divided each subsequent timepoint by this average to adjust the data to time = 0. Two-way analysis of variance (ANOVA) tests to compare levels of HMGB1 protein were performed with GraphPad Prism 5 software using a p value of < 0.05 as a cutoff for

significance. Parameters measured in ANOVA tests were: Treatment (control temperature vs. cycling temperature), Time (sampling intervals) and Interaction (the interaction of Treatment and Time).

DNA sequence analysis of the putative promoter region of the *hmgb1* gene was performed using a number of tools available online. For a synopsis of all programs employed, as well as parameters/thresholds used, see Table 2. GC content was analyzed using the OligoCalc program (Kibbe 2007). Putative transcription factor binding sites were identified using the vertebrate matrices in the TFSearch program (Heinemeyer et al. 1998). Putative CpG islands were identified using the CpGPlot program (Larsen et al 1992). Putative promoter sites were identified using the Berkeley Drosophila Genome Project (Reese et al. 2001). Only putative promoters within 2100 base pairs of the start of the coding region are annotated in Fig. 7.

<p>OligoCalc: http://www.basic.northwestern.edu/biotools/oligocalc.html (Kibbe 2007)</p> <p>TFSearch: http://www.cbrc.jp/research/db/TFSEARCH.html (Heinemeyer et. al. 1998) Parameters: ID threshold = 90</p> <p>CpGPlot: http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html (Larsen et. al 1992) Parameters: Window = 100; Step = 1; Obs/Exp = 0.6; MinPC = 50; Length = 200</p> <p>Berkeley Drosophila Genome Project: http://www.fruitfly.org/seq_tools/promoter.html (Reese et. al. 2001) Parameters: ID threshold = 0.95</p>
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Table 2. Online programs used to analyze the 5' upstream region of the *hmgb1* gene in *A. limnaeus*.

Results

HMGB1 protein levels and subcellular localization

A principle band was detected at ~27 kDa using the HMGB1 protein antibody employed in this study (Figure 3, 4). HMGB1 protein content of whole liver lysates quantified from these blots is presented in Figure 4. Temperature cycling had no significant effects on HMGB1 protein levels ($F = 1.150$, $p = 0.2943$). Additionally, time was not a statically significant variable ($F = 0.08716$, $p = 0.9856$), nor was the interaction of time and temperature ($F = 0.5409$, $p = 0.7072$). The subcellular localization of HGMB1 protein as assessed by western blots (Figures 6 and 7) and immunohistochemistry (Figure 8) does not support a strong change in subcellular localization of the protein in response to temperature cycling. Most of the HMGB1 protein is found in the cytoplasmic compartment in liver tissue of *A. limnaeus* (Figures 6 and 8). However, our methods do not allow for a strict quantification of the subcellular distribution of this protein. Although overall patterns of cytoplasmic subcellular localization did not change in a statistically significant manner, the effect of temperature cycling is significant ($F = 4.605$, $p = 0.0476$) for the nuclear fraction. This could suggest a muting of the natural translocation of HMGB1 into the nucleus observed in control fish at around 9:00 at night. (Figure 7b).

I believe that the recovery of HMGB1 from the subcellular fractions was complete and that the data represent true amounts of this protein in each fraction. HMGB1 possesses a large number of charged amino acid residues and it is quite soluble in salt. Purification of HMGB1 can be carried out using 0.35 M NaCl and the protein is soluble in 2% trichloroacetic or 5% perchloric acid (Goodwin and Sanders 1973).

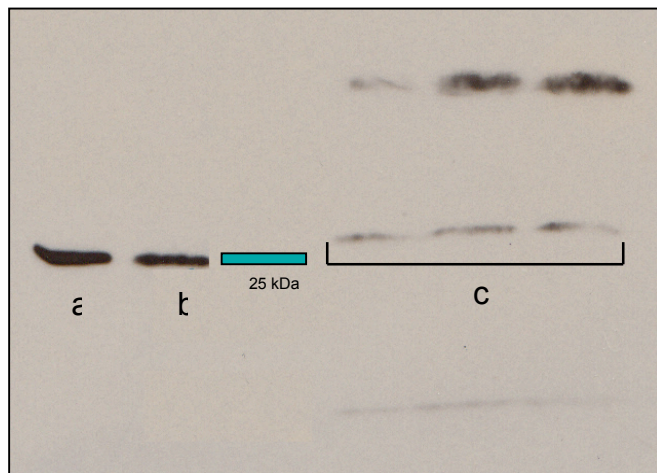


Figure 3. Immunoblot of HMGB1 positive control and experimental samples. HeLa cells (a), control histones (b) and *Austrofundulus limnaeus* liver cells (c). Blot also shows both high and low molecular weight nonspecific binding.

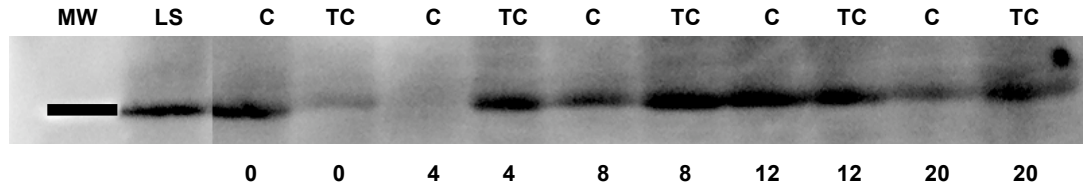


Figure 4. Representative immunoblot showing detection of a band at approximately 25 kDa in liver tissue isolated from mature *Austrofundulus limnaeus*. The numbers under the photograph indicate hour at which the sample was taken. MW, 25kDa molecular weight standard; LS, standard liver sample present on every blot; C, control; TC, temperature cycling.

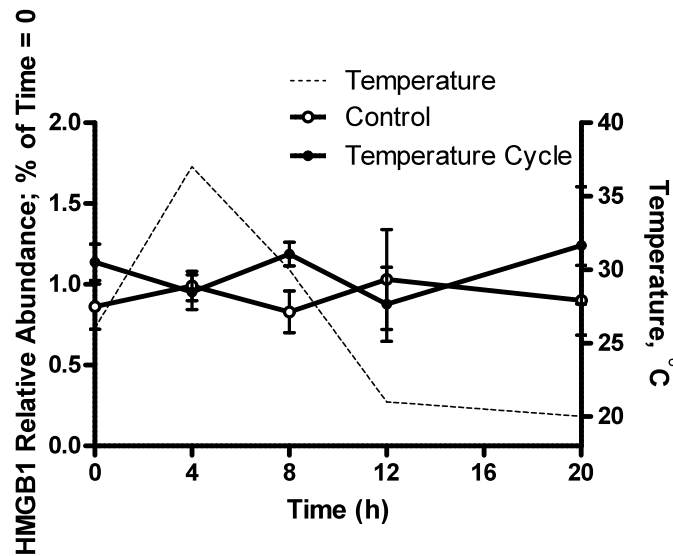


Figure 5. HMGB1 expression in *Austrofundulus limnaeus* livers exposed to temperature cycling. Samples are expressed as a percentage of control samples at each timepoint. Symbols represent means \pm S.E.M. ($n = 4$). Water temperature of the temperature cycling tank is represented as a dashed line. No significant differences in total HMGB1 levels were detected (ANOVA, $F = 2.763$, $p = 0.0771$.)

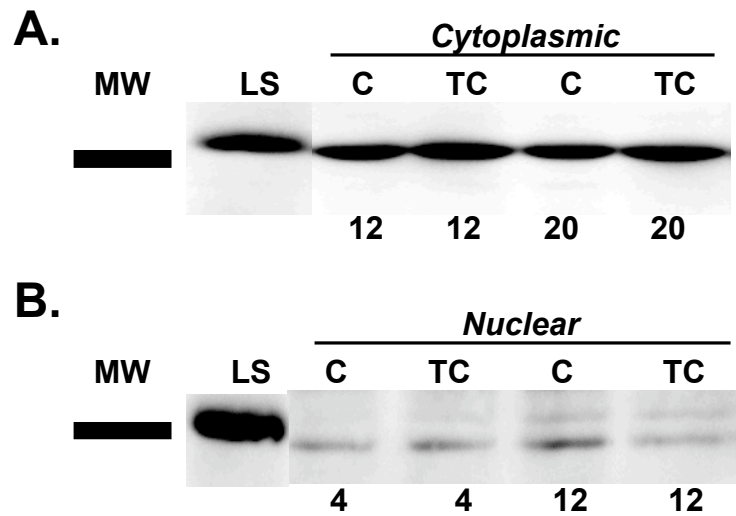


Figure 6. Representative immunoblot showing detection of HMGB1 in crude (A) cytoplasmic and (B) nuclear fractions of *Austrofundulus limnaeus* livers. The numbers under the photograph indicate the time point in hours. MW, 25kDa molecular weight standard; LS, standard liver sample present on every blot; C, control; TC, temperature cycling.

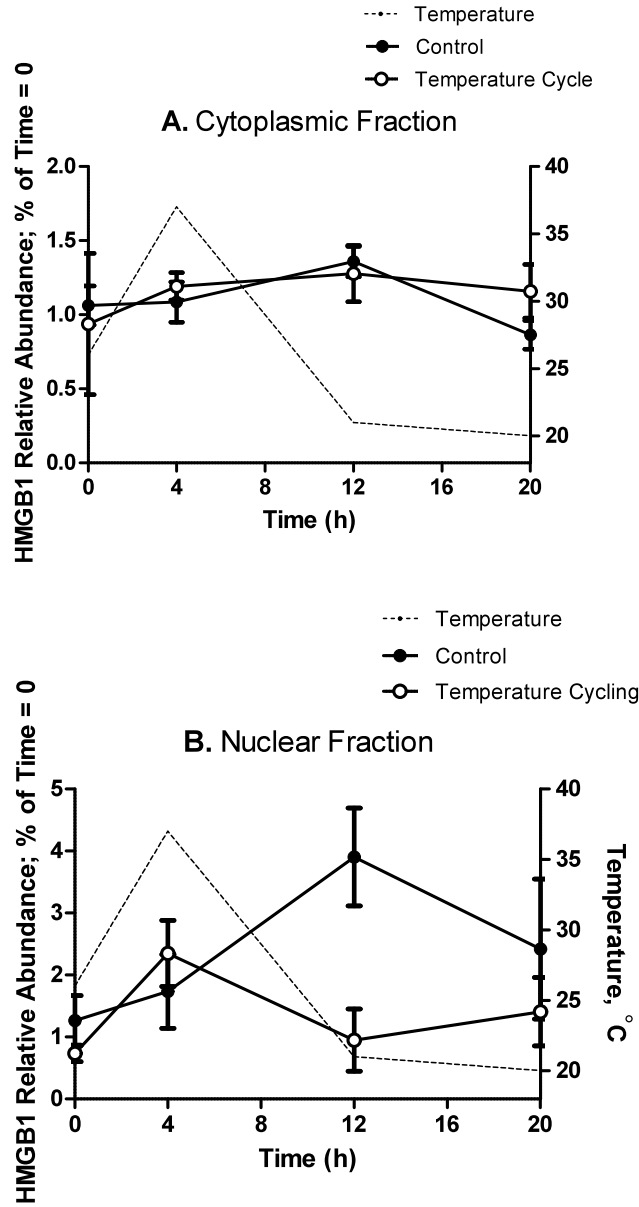


Figure 7. HMGB1 expression in (A) cytoplasmic and (B) nuclear subcellular fractions of *A. limnaeus* livers. Symbols represent means \pm S.E.M. ($n = 3$). Two-way ANOVA indicates a significant interaction between temperature cycling and time for the nuclear fraction. (Two way ANOVA $F = 4.605$, $p = 0.0476$.)

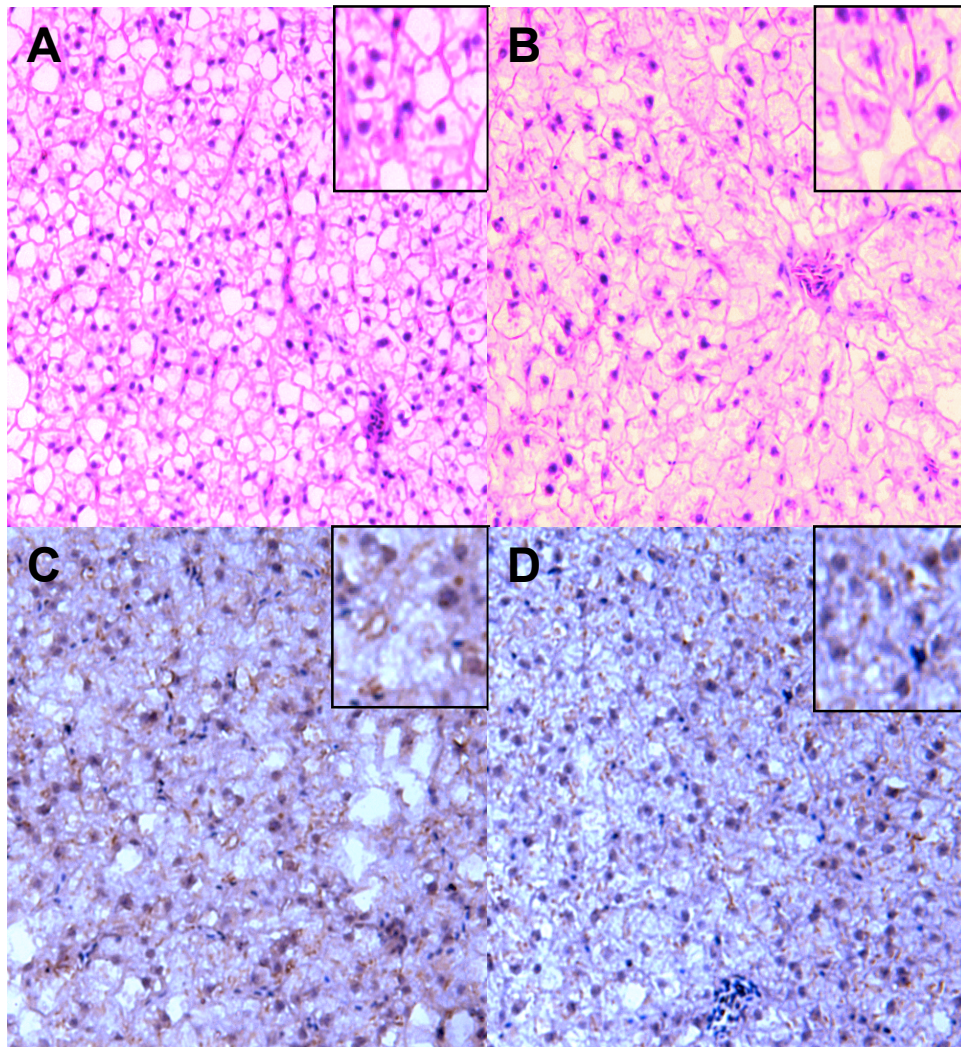


Figure 8. Immunohistochemical staining of *A. limnaeus* liver tissue subjected to control (A,C) and temperature cycling (B,D) treatments. DAB staining (A,B) indicates both nuclear and cytoplasmic staining for HMGB1. Hematoxylin and eosin stained (C,D) sections are provided to illustrate the overall tissue and cellular morphology.

Isolation and sequence analysis of hmgb1 5' flanking region

A 4,552 bp region of DNA (Figure 9) located directly upstream from the previously described *A. limnaeus* cDNA was isolated and sequenced. This sequence has a small region with 1% overlap with human the HMGB2 sequence in the non-redundant GenBank sequence database as assessed using the NCBI basic local alignment search tool (nucleotide BLAST). When tested for sequence similarity (BLAST search) specifically against the zebrafish genome there was a 6% overlap with the Chromosome 10 genomic scaffold, an area known to contain the *hmgb1* gene. The entire upstream sequence has a GC richness of 41%, and two predicted CpG islands from 1516-1969 bp and from 2603-2901 bp. TFSearch identified the presence of a number of putative transcription factors within an identification threshold set at 90 (Figure 9 and Table 2). However, within an identification threshold set at 85 there are an additional 5 putative binding sites for heat shock factors 1 and 2 (HSF1 and 2) within 1,000 base pairs of the beginning of the coding sequence. There are 3 possible promoter start sites in the sequence occurring at 2132 bp, 2025 bp and 1313 bp.

1350 CCTTTTCCTCAACGAGAAGCCTATGCGCCATTATTACTGCTTGTGTGCGAC
CdxA

1300 GCGGCTCAGGACT **TACAGCAGCGTTTAATACGGGGGAAACGTACGGGATG**
CdxA

1250 **GAAAAGGGAGCGT**TTTAAACGCGTTTAGCGAGTTATTTACGGGAAAAAAT
CdxA

1200 AAACAAAAAAACAAAGACGCGCACGGGAGGAGGAGTGTGCTCGCATTA
SRY HNF-3b

1150 CGTTGCACTTTTCAGGGTTATTTTTTCAATAAAAGCGACTTTTTGTGACA
TATA

1100 TTTTGGCTGTTTGTGTGCGAACTTTATTTCTTCAATAAAACAATAAAAAACA
Sox-5

1050 CTTATTGGATTTTTGTTTTTTGACACTTTAGGCAGGGGTTTTATTTATTT
CdxA

1000 TTCACCAAAAACACAGTTTTTAAACAAAATGCACGGATTGAAGTAAGAGC
SRY SRY

950 TGAACTTTTTTGGAAAACCTACGTCAGTTATGCAGACAATAAGGTGGCTT
C/EBP

900 TAATAAAAACAAAATTACACTTTTTTAAATTATTACTATTTTTTTTAAATC
CdxA SRY CdxA

850 TCTGATTTAATGGGATTAGGCAGCTTTAAGCCACCATAATGAGAGCAGTC
CdxA Oct-1

800 CCTGCAGTTTTACATAAGGGCAATAAATGAAGTCATTTTCATGGCTTTAG
750 CTTTATATTTGCAGTAGGGGTGTAAGAAAATATCGATACACTGAAATATC
CdxA

700 GCGATATTTCAATTTCTCGATACTGTATCGATATTCTAAAGCCCTGTATCG
650 ATTTTTTTTAATTAAGTTAAAAATGTCAACCTTCTTGCCAACCGTTTATTTT
S8

600 TATGTTGTAGTTCTGCCTCTGACCACTAAATTAAGTTAAAATGTCAACCT
CdxA

550 TCTTGCCAACCGTTCATTTTTTATGTTGTAGTTCTGCCTCTGACCACTAGT
CdxA

500 TGGCAGCAGTGAGCCATCCATTTGGCTCCTCTACCTCTTGAGCGCCAGAG
450 AGTAAGGGTAGCATTAGCAATGTAAGTAAACATTAGCATTTTTTTTTTTTC
400 GTAATAGTTTTTTATTTAATAGAATTTTTTGGACAAAAAAATCGCAATATA
CdxA

350 TCGCAATATATCGGGATATATCTTATCGTGACCCCTCGGTTATGATATGT
300 ATCGTATCGCCAAATTCTTACCAATACACAGCCCTAATTTGCAGCAAGGA
250 GGGTGAGTGCCAAATACAAGGAATAAGATAATCAGAGCGCTGTTAGGGT
Evi-1

200 ACATTCTAGTTAAACAGAAACCTCTATTCGTAGTGTTTTATCCACTTTA
SRY

150 GCAGGCACAGACTTAATCTGTACCTAAACGTTTTTTTTTTTTTTTTTTAG
100 ATACTGCTATCAAGATGGGGAAGGATCCAATAAGCCGAGAGGGAAGATG
GATA-1

50 TCTTCCTACGCGTATTTTTGTTTCAGACCTGCCGGGAAGAGCACAAGAAGAT
MZFI

Figure 9. Nucleotide sequence of the 5' region of the *A. limnaeus* hmgb1 gene from -4552 to the start of the current published sequence (GenBank accession number: CK817293.1). Putative CpG islands are in lowercase text. Putative transcription factors are underlined and labeled. Putative promoters (within 2100 bp's of the coding region) are in bold italic font. The putative start codon (ATG) for the coding region is in bold font.

<u>Transcription Factors by Role</u>
Stress
CREB
SREBP (also Lipid Homeostasis)
Oct-1
C/EBP
Development
CdxA
SRY
Nkx-2
Sox-5
S8 or Prx2
Cancer/Cell Cycle
GATA-1
Tal-1 a/b
E4BP4
PBX-1
Evi-1 (also Inflammation and Circadian Clock)
MZF-1
HNF3b or FoxA

Table 3: List of transcription factors that appear in upstream region of the *hmgb1* gene of *A. limnaeus*. Transcription factor predictions were generated by TFSearch program.

Discussion

The results presented in this thesis examine the cellular patterns of HMGB1 protein expression in *A. limnaeus* as well as the sequence and structure of the 5' upstream region of the *hgmb1* gene. In my experiments, I did not find a correlation between *hgmb1* transcript levels and HMGB1 protein levels in liver tissue. The high cytoplasmic localization of HMGB1 within killifish livers in my experiments is in line with other studies, however my nuclear localization data do not match with other systems. In the following discussion, I will describe both the (1) HMGB1 protein expression patterns in response to temperature as well as (2) the implications for the sequence of the upstream promoter region. These two pieces of evidence shed light on how the *hgmb1* gene is expressed in response to large-scale alterations in temperature, and more generally in response to cellular stress.

The HMGB1 Protein in *Austrofundulus limnaeus*

Subcellular Distribution

Although overall levels of the HMGB1 protein are not significantly altered by temperature, changes in subcellular localization could play a role in regulating the activity of this protein in response to temperature. To determine the subcellular localization of HMGB1, I conducted western blots on crude cytosolic and nuclear fractions of liver tissue. The data illustrate two major points about the distribution of HMGB1 in response to time and temperature. First, most of the protein is found

in the cytoplasm. Second, in control fish, a seemingly natural translocation of HMGB1 into the nucleus occurs at roughly 9:00 pm (Fig. 7b). This translocation appears to be muted in fish exposed to temperature fluctuations (Fig. 7b). However, the functional significance of this change in subcellular localization is currently not known. Additionally, I did not observe cytoplasmic HMGB1 levels to change, but it is possible that the protein was translocated into different compartments within the cytoplasm, such as secretory lysosomes in preparation for export. To further investigate this idea blood samples would have to be tested for HMGB1 protein to look for active export of HMGB1 protein in response to temperature fluctuations.

Transcript vs Protein Levels

My data suggest that HMGB1 protein levels differ from *hmgb1* transcript levels in *A. limnaeus*. The hypothesis of Podrabsky and Somero (2004), that the HMGB1 protein is a global regulator of transcription, does not seem valid for *A. limnaeus*. mRNA transcript levels for *hmgb1* are reduced compared to control levels just after a temperature insult or “heat shock”, and then increase to just above control levels following the heat shock (Fig.1). This pattern could be interpreted as a reduction in transcription due to heat shock, followed by a recovery as temperatures cool off. This explanation coordinates with general transcription patterns observed during heat shock. During a heat shock event, most genes are not translated except for a select group of heat shock proteins/elements (Lindquist

1986). After the temperature insult, many genes are turned on to help facilitate the cell's recovery (Lindquist 1986). HMGB1 is strongly upregulated after heat shock and could serve as part of the “clean up” machinery employed after a heat insult. However, it should be noted that I did not perform any experiments on the *in vivo* kinetics of HMGB1 protein turnover, and so I am unable to comment on the rates of synthesis or degradation of this protein in killifish hepatocytes. Though it should be noted that the disparity observed between HMGB1 protein abundance and transcript levels could suggest that HMGB1 protein displays very high rates of degradation and turnover *in vivo*. Alternatively, the transcript for this gene could also be very heat labile. Further investigation will be required to better understand the lack of coordination between protein and transcript levels for this gene.

The lack of a correlation between protein and transcript levels are somewhat surprising as for many genes the level of a given protein will match its mRNA transcript level. However, there are many examples that show divergences in expression levels of mRNA transcripts and their protein products. Many post-translational modifications made to proteins (acetylation, glycosylation, methylation etc.) could help explain this difference (Laurent et al. 2010) by altering protein stability or function independently of transcript levels. Additionally, proteins can be degraded quickly such that to calculate a protein's concentration one would use the equation: $t_{1/2} = (A_{\text{total}}/2) / ((SR \times t_{\text{dulp}} - A_{\text{total}}) / t_{\text{dulp}})$ where $t_{1/2}$ is the biological half life of a protein, A_{total} is the absolute protein amount, SR is

synthesis rates of the protein, and t_{dup} is the duplication time of the cells (Gerner et al 2002). More simplistically this equation could be described as the change in a protein's concentration over time is equal to the rate of translation minus the rate of degradation (Greenbaum et al. 2003). Moreover, there are many studies that observe disparate protein and mRNA levels. A study in yeast demonstrated that while some protein levels remain constant, their mRNA transcripts can change as much as 30-fold when grown until early log phase (Gygi et al. 1999). In both yeast and *E. coli*, only 73% or 47%, respectively, of the variance seen in protein abundance can be explained by mRNA abundance (Lu et al. 2007). In a study that spanned seven species and four kingdoms, researchers discovered that steady-state abundances of certain proteins are more correlated across taxa than with their own mRNA levels (Laurent et al. 2010). This result could suggest that protein abundances are evolutionarily conserved despite diverging mRNA levels (Laurent et al. 2010). Though *hmgbl* mRNA levels and protein contents were not found to be related in killifish livers, it is clear that the mRNA transcript levels are remarkably sensitive to temperature (see discussion below).

A study by Wang and colleagues provides one of the only comprehensive looks at both the mRNA for the *hmgbl* gene as well as the protein levels of HGMB1. Their study employed *in vitro* culture of murine (mouse) macrophages coupled with a sub-lethal dose of lipopolysaccharide (LPS). When these cells were stimulated with LPS, the mRNA levels did not change over a 24hr observation period (Wang

et al. 1999). However, the protein was strongly induced. At 0 - 4hr (post LPS insult) they measured $\sim 0 \text{ ug}/5 \times 10^6 \text{ cells}$, at 8hr they measured $\sim 8 \text{ ug}/5 \times 10^6 \text{ cells}$ and at 24hr they measured $\sim 12 \text{ ug}/5 \times 10^6 \text{ cells}$. From these data, they deemed it unlikely that HMGB1 release is related to increased gene transcription. In further support of this “hypothesis” they used a pulse labeling technique (with ^{35}S -methionine) to measure the turnover rate of HMGB1 which showed that the radioactivity was only incorporated into the proteins after 12hr. They postulated that the HMGB1 released between 0 and 12hr, came from a preformed pool.

Study	Cell Type	Lethal/Sub-Lethal Dosage	mRNA	Protein
Wang	Mouse: macrophages	Sub-Lethal LPS	No Change	↑ 6-8hr after insult, peaks 24hr after insult
Sass	Mouse: liver	Lethal LPS	↑ after 1hr, ↓ after 6-12hr, baseline 12-24hr	Not measured
O'Connor	Rat: brain	Sub-Lethal LPS	No Change	Not measured
Todani	Rat: liver, plasma	Sub-Lethal Temperature	Not measured	↑ 1hr after hyperthermia
Gracey	Common Carp: gill, kidney, brain, heart, muscle, liver	Sub-Lethal Temperature	↑ with cold temperature	Not measured
Vergauwen	Zebrafish: Liver	Sub-Lethal Temperature	↑ with cold temperature, ↓ with warm temperature	Not measured
Truebano	Antarctic Clam: gill, mantle, siphon	Sub-Lethal Temperature	↑ with warm temperature	Not measured
Podrabsky	Killifish: liver	Sub-Lethal Temperature	↑ with cold temperature, ↓ with warm temperature	Not measured

Table 4. Studies involving the transcriptional (mRNA) and translational (protein) responses of HMGB1 in a variety of organisms and experimental setups.

To date, there are a handful of studies that have characterized the mRNA profiles of *hmgb1* in response to non-lethal environmental temperature perturbations and LPS injections to induce a febrile response (See Table 4(1-83)). In studies of zebrafish and carp, the mRNA was up-regulated in response to acclimation at cold temperatures (Vergauwen et al. 2010, Gracey et al. 2004). Specifically in zebrafish, the transcripts were also down-regulated after acclimation at warm

temperatures. In the case of Antarctic clams, the *hmgb1* transcript was positively correlated with an increase in acclimation temperature from 0°C to 3°C (Truebano et al. 2010). A single study on temperature change in rats showed that HMGB1 protein levels were increased in the liver and plasma in response to 1 hr of hyperthermia of 40°C (Todani et al. 2010). Intracerebroventricular injections of HMGB1 into rats resulted in increased body temperatures and branded HMGB1 as a pyrogen (O'Connor et al. 2003). Some studies suggest that inflammatory cytokines (of which HMGB1 is one) act as mediators in fever, modulating and interacting with the point of initiation of the heat shock response and stress response (Hasday and Singh 2000). This interaction between inflammatory mediators and body temperature could allow for fine control of the stress response (Hasday and Singh 2000). For example, if an organism experiences a fever due to infection, induction of the stress response would be beneficial. However, if an organism is simply experiencing slightly elevated environmental temperatures, the induction of the stress response may not be warranted.

Two studies have measured *hgmb1* mRNA in response to LPS stimulation. In one experiment, researchers administered lethal doses of LPS to mice and assayed the livers for expression of *hmgb1*. They found that mRNA was up-regulated 1 hr post LPS insult, down-regulated 6-12 hr post insult and finally returned to normal baseline levels after 12-24 hr (Sass et al. 2002). Although these results do show marked changes in mRNA levels for *hmgb1*, they are not surprising

given that the animals experienced a lethal dose of stimulant. However, in another study in rats, a sub-lethal dose of LPS did not result in changes in mRNA levels (O'Connor et al. 2003).

From these experiments, we can start to draw some overall conclusions of what *hgmb1* transcript and HMGB1 protein may be doing in these various systems in response to temperature and bacterial stress and how they compare to the killifish model. It appears that in mammals, HMGB1 mRNA levels are affected only by very strong stimuli, but there is a clear correlation between increased body temperatures and induction of HMGB1 protein expression. Conversely, in killifish, the mRNA levels decline quite drastically when a warm sub-lethal temperature insult is applied, and protein levels do not appear to be associated with environmental temperature. Although LPS and temperature represent two different cellular assaults, it appears that killifish *hgmb1* mRNA levels are more labile than that of rodents. The data from the temperature affected aquatic organisms (carp, clams and zebrafish), further support the idea that these mRNA's may be more sensitive to environmental perturbations. Although none of the studies employed a cycling temperature regime such as with the killifish, the transcriptional response of *hgmb1* in all of these species was significant and, in carp and zebrafish, consistent with killifish; a decrease in mRNA levels in response to high temperature and a decrease in response to warm temperature. (Truebano et al. 2010, Gracey et al. 2004, Vergauwen et al 2010, Podrabsky and Somero 2004). From

these data I suggest that the *hmgb1* mRNA may be playing a role directly as a temperature sensor in these aquatic organisms.

The highly consistent response of *hmgb1* transcript levels to changes in temperature may suggest a role for the mRNA itself as a cellular temperature sensor. If the mRNA plays this role, there are several possible ways that mRNA transcript levels could be used to measure or monitor cellular temperature. First, this molecule could be acting as an RNA thermometer. Second, differential degradation of the transcript in response to temperature could explain the observed patterns and alter activity of the mRNA. Third, alterations in the levels of *hmgb1* transcript could be a mechanism to alter the activity of a critical temperature-sensitive micro RNA.

RNA temperature sensors are known to be important in prokaryotic systems where they operate at the post-transcriptional level by sensing specific temperatures and then conveying their signal to the translation machinery (ribosome) via a conformational change (Chowdhury et al. 2006). RNA “thermometers” work through the destabilization of a hairpin loop between the AUG start codon and the Shine-Dalgarno sequence. Both elements are located near to each other in the 5’ untranslated region (UTR) of bacterial genes. When the temperature is elevated enough, this hairpin structure is destabilized allowing the ribosome to bind and initiate translation (Klinkert and Narberhaus 2009). These RNA thermometers allow the cell to induce a highly dynamic response to stressors very quickly

(Narberhaus 2006). Although they are not as well characterized in eukaryotes, RNA temperature sensors are present and represent an evolving frontier in gene regulation (Thore and Frick 2008, Wachter 2010). Because RNA thermometers are mostly thought to work through translational control of gene expression, I feel it is unlikely that this mechanism is active in liver tissue of annual killifish. For this mechanism of action, I would have to invoke a high turnover rate of the HMGB1 protein in response to temperature that is compensated by changes in translation of the mRNA. This may be the case, but it does not explain the large-scale changes in mRNA levels observed. If the *hmgb1* transcript is acting as an RNA thermometer, it would have to be associated with regulation of transcription, and this would represent a novel finding in the regulation of gene expression in eukaryotes.

In *A. limnaeus*, the transcriptional response of *hmgb1* to temperature could be explained by increased degradation of the mRNA in response to high temperature. Ribonucleases (RNases) are a class of molecules that both process and degrade RNA (Tomecki et al. 2010). They play an important role in regulating the expression of genetic information throughout all the kingdoms. In a bacterial example, a particular RNase was found to be catalytically dependant on temperature. In *E. coli*, the mRNA for *cspA* possesses a 5' loop (at cold temperatures), which prevents the mRNA from interacting with RNase E and becoming degraded (Hankins et al. 2007). This loop is more stable at lower temperatures and thus the half-life of the mRNA is longer at lower temperatures (47s at 30°C) as opposed to higher temperatures (10s at 37°C) (Goldenberg et al.

1996). A mechanism similar to this could be at play in the regulation of the killifish *hmgbl* mRNA where we see a loss of mRNA in response to high temperatures. It could be that the *hmgbl* mRNA possesses a modification that at high temperatures is recognized by an RNase; leading to degradation and a significant drop in transcript level.

Micro RNA's (miRNAs) are small molecules of RNA (~22 nt) that can induce gene silencing, suppress protein translation and cleave mRNA (Bartel 2004). Research indicates that miRNAs and mRNA can be produced from a single primary transcript. Often the miRNA sequence is located in an intron, and is only spliced out of the mRNA when excision of introns occurs (Kim et al. 2007). So essentially, for a given gene, the mRNA, the miRNA and the protein are all encoded by the same primary transcript. In killifish, the increase in mRNA transcript could be the cell actually attempting to produce miRNA for regulatory purposes.

Future Studies for Proteomics

To more thoroughly explore the expression of the *hmgbl* gene in response to temperature, there are a number of future studies that could be pursued. Perhaps the time scale used in my current experiment was not long enough to see the true effects of temperature on HMGB1 abundance. Separate fish held at 20°C and 37°C would provide insight into how the protein may respond to long periods of “stressful” temperatures. For laboratory experiments, two-dimensional (2D) gel

electrophoresis could aid in pinpointing the exact covalent modifications that are possible with this protein. Further, the use of fluorescent labeled 2D gels would allow both the control and experimental samples to be visualized on the same gel and thus eliminate the “warping” of traditional 2D analysis. Additionally, the use of an immobilized pH gradient 2D gel could also aid in the visualization of HMGB1. The in-cell kinetics of HMGB1 could be visualized using a “pulse-chase” experiment. Cells would be “pulsed” with a radioactively labeled substance that would become incorporated into the HMGB1 proteins. After a “chase” with the same non-radioactively labeled substance, the level of radioactivity is measured in HMGB1 after a certain time. The levels of radioactivity (measured after the elapsed time period) determine the synthesis and degradation rates of the protein. Another way to establish protein synthesis/degradation rates in the cell would be to block the proteasome and monitor the cell for a buildup of HMGB1. The chemical MG132 is a known proteasome blocker (Genschick et al. 1998). If it is administered and there is an observed build up of HMGB1 protein over the sampling time scale, then it would suggest that the protein is constantly being synthesized and degraded. If there is no build up of protein, it would mean that the cell maintains steady state levels of HMGB1.

5' Upstream Region of the *hmgb1* Gene

HMGB1 is a critically important protein due to its ability to directly interact with DNA as well as influence the mammalian inflammatory response. My analyses of the 5' upstream region of the *hmgb1* gene illustrate a number of putative regulatory elements. Although the role of HMGB1 in *A. limnaeus* is not yet fully elucidated, identifying the structure of the gene will allow us to better understand the highly temperature-dependent nature of its transcription. The following discussion will focus on the second part of my thesis regarding the 5' upstream region of the *hgmb1* gene.

Expression of the human *hmgb1* gene is controlled by a very strong TATA-less promoter (Lum et al. 2001). Additionally, the gene contains both a silencer and multiple enhancers that allow for a wide range of expression patterns. Although this analysis of the upstream region for the killifish *hmgb1* gene did not include functional promoter assays, the TFSearch program was utilized to identify putative *cis*-acting elements, or transcription factors, in the sequence. Some of the transcription factors that appear in the killifish upstream region are known to be involved with various cellular stress responses.

A number of stress-inducible transcription factor binding sites are located in the upstream region of the *hmgb1* gene. One of these transcription factors is octamer transcription factor 1 (Oct-1), which appears to be a general sensor of stress. In Oct-1-deficient fibroblasts, the cells can become hypersensitive to stressors such as γ radiation and hydrogen peroxide (Tantin 2005). In a study on

heavy metal induced cellular stress, sex determining region Y protein (SRY) as well as Oct-1 were found to be upregulated (Glahn et al. 2008). Although caudal-type homeobox domain transcription factor (CdxA) is one of the most common promoter elements found in human DNA (Bajic et al. 2003); in *C. elegans*, it functions as a general regulator of transcription (Anokye-Danso et al 2008). CdxA in zebrafish hepatocytes stimulates genes involved in wound repair (Chiou et al. 2006). In human carcinomas, CCAAT enhancer binding protein (C/EPB) is implicated in mitochondrial stress-mediated activation of certain target genes (Biswas et al 2005). Additionally, C/EBP's are able to transactivate stress response genes in response to injury in rats (Gilpin et al. 1996). Sterol regulatory element binding protein (SREBP) is a transcription factor bound to the endoplasmic reticulum (ER) membrane whose main role is to upregulate genes involved in fatty acid and cholesterol synthesis (Colgan et al 2007). However, during ER stress (a state where misfolded proteins cause the “unfolded protein response” to occur which temporarily inhibits translation), SREBP is upregulated suggesting that it plays a role in regulating the stress response (Colgan et al 2007). cAMP response binding element protein (CREB) is a well documented stimulus-induced transcription factor and is important in mediating cell responses to extracellular stimuli (Shaywitz and Greenberg 1999). CREB is a direct target of the cAMP signaling pathway as well as other stimulus-inducible pathways triggered by molecules such as peptide hormones and growth factors (Shaywitz and Greenberg 1999).

Future DNA Studies

Future studies of the upstream region of the *hmgb1* gene should include the use of a reporter assay system. Reporter assays require the putative promoter region to be cloned into a vector with a reporter gene and thus allow for the functional activity of the promoter to be tested. To pinpoint the exact area of promoter activity, nested deletions of the sequence take place to create different fragments. In addition to the upstream promoter region, the introns and exons can be assayed in this same manner to reveal possible silencer and/or enhancer elements.

Overall Conclusions

In this study, I did not find a correlation between mRNA transcript levels and cellular protein content for the HMGB1 protein. The hypothesis of Podrabsky and Somero (2004), that the HMGB1 protein is a global regulator of transcription, does not seem valid for *Austrofundulus limnaeus*. Although transcript and protein levels do not correlate, this work is still critical for understanding the possible role that HMGB1 might be playing in response to temperature stress in killifish. *hmgb1* expression patterns do not match those of a typical “heat shock” responsive gene, and thus the HMGB1 protein is very likely not a key component of the heat shock response in this species. This conclusion is supported by the lack of heat shock

response elements in the upstream region of the *hmgb1* gene. However, the fact remains that the transcription of this gene is highly responsive to temperature, data which are supported by previous studies in carp, Antarctic clams and zebrafish. To my knowledge, *hmgb1* is the only gene yet identified with such a marked transcriptional response to alterations in temperature. Thus, I suggest that the *hmgb1* transcript, and not the protein, may be acting as a cellular temperature sensor. Future studies should focus on the role of the *hmgb1* transcript in possibly monitoring or sensing the cellular thermal environment and transducing this signal to help coordinate a cellular homeostatic response.

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